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# A Novel Flow Cytometric Steroid Hormone Receptor Assay for Paraffin-Embedded Breast Carcinomas: An Objective Quantification of the Steroid Hormone Receptors and Direct Correlation to Ploidy Status and Proliferative Capacity in a Single-Tube Assay

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Semiquantitative estimation of steroid hormone receptors by immunohistochemistry applied to paraffin sections is common practice in surgical pathology. Flow cytometric (FCM) analysis of estrogen receptor (ER) and progesterone receptor (PR) levels provides a faster and more objective quantitative assay. However, a major problem in such FCM analyses of solid tumor samples is the admixture of tumor cells with normal epithelial, stromal, and inflammatory cells. The aim of the underlying study was to investigate the applicability of a recently developed multiparameter flow cytometric methodology for the accurate estimation of the fraction of steroid hormone receptor-positive tumor cells and to explore whether this multiparameter approach allows the detection of specific, clinically relevant subsets of tumors, based on a combination of ploidy level, steroid hormone receptor status, and cell cycle characteristics. For this purpose, samples of 42 breast cancer patients, from which routine immunohistochemistry for ER and PR also was available, were analyzed. From each case, a cell suspension was prepared from the paraffin block by applying a heating and short pepsin digestion step to 50- $\mu$ m-thick sections. These cell suspensions were double-immunostained for cytokeratin to identify the epithelial cells, and ER or PR, whereas DNA was quantitatively stained with propidium iodide using an optimized protocol. In the entire group of breast tumors, the percentages of ER- and PR-positive cells were registered in the

epithelial subfraction, in combination with DNA ploidy and S phase fraction (SPF). A significant correlation was found between the fraction of hormone receptor-positive cells as found by the immunohistochemical and FCM procedures. For ER, a correlation coefficient of  $r = .87$  was found, and for PR  $r = .62$ , both  $P < .0001$ . It became clear that all the diploid breast tumors had more than 30% tumor cells positive for ER with a SPF lower than 10%, whereas aneuploid tumors contained on average a smaller percentage of steroid hormone receptor-positive cells, and simultaneously an SPF greater than 10%. Our results show that this multiparameter FCM analysis allows an objective and reproducible quantification of the fraction of steroid hormone receptor-positive cells in the relevant epithelial cell compartment in relation to DNA ploidy status and proliferative capacity in a single-tube assay. HUM PATHOL 31:584-592. Copyright © 2000 by W.B. Saunders Company

**Key words:** estrogen receptor, progesterone receptor, cytokeratin, multiparameter FCM.

**Abbreviations:** ER, estrogen receptor; PR, progesterone receptor; FCM, flow cytometry; SPF, S-phase fraction; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; RPE, r-phycoerythrin; Ig, immunoglobulin; PI, propidium iodide; RFI, relative fluorescence intensity; RT, room temperature; H&E, hematoxylin and eosin.

The levels of estrogen and progesterone receptor (ER and PR, respectively) are important parameters to predict short-term prognosis and the response to endocrine therapy of breast and endometrium cancer. Therefore, the analysis of steroid hormone receptor content is widely used in the management of these hormone-dependent malignancies.<sup>1-11</sup> The expression of ER and PR can be determined by quantitative assays, such as ligand binding assays and immunochemical assays, by semiquantitative immunohistochemistry, or by flow cytometry (FCM).<sup>12</sup> However, the heterogeneous cell

composition of most tumor samples is one of the limiting factors for both quantitative and semiquantitative receptor assays. Multiparameter FCM analysis of cytoplasmic markers (cytokeratin), hormone receptors (ER or PR), and DNA content facilitates the (semi)quantitative measurement of hormone receptors in the relevant subpopulation of cells by focusing on the epithelial cells. Furthermore, receptor content can be analyzed in relation to DNA ploidy and growth potential, which is reflected by the percentage of tumor cells in the S-phase of the cell cycle.<sup>13</sup> Both DNA ploidy and the S-phase fraction (SPF) have been shown to be important prognostic parameters in cancer. However, this approach is mainly limited to prospective studies, because fresh, unfixed tissue samples are normally required.

In earlier studies, however, we showed that formalin-fixed, paraffin-embedded tissue samples can be processed for multiparameter FCM.<sup>14-16</sup> Recently, we modified the method by including a heating step, applied to 50- $\mu$ m-thick deparaffinized, rehydrated tissue sections

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for 2 hours at 80°C before a short and mild pepsin digestion. This leads to improved cell recovery and high-resolution DNA histograms.<sup>17</sup> Furthermore, heating of tissue sections resulted in improved results of immunocytochemical staining procedures applied to the cell suspensions because of improved epitope retrieval. In the current study, we applied this new multiparameter FCM technique to the quantification of the steroid hormone receptor-positive cell fraction in the relevant epithelial cell population from archival material of breast carcinomas. To establish optimal conditions for this assay, we analyzed the effects of duration of enzymatic digestion and incubation with the primary antibody on immunoreactivity with the different antibodies directed against the steroid hormone receptors, and investigated the intra-assay and interassay variability. The results of these trivariate FCM analyses were compared with the immunohistochemical analyses on tissue sections of the same paraffin-embedded tumor blocks.

## MATERIALS AND METHODS

### Breast Carcinoma Samples

Samples from the primary tumors were obtained during surgery from 42 breast cancer patients undergoing either elective mastectomy or lumpectomy. These tumors were diagnosed according to the World Health Organization classification and graded according to Bloom and Richardson.<sup>18</sup> Tumor stage was assigned according to the TNM classification. The tumors were classified as infiltrating ductal carcinoma ( $n = 35$ ), lobular carcinoma ( $n = 6$ ), and mucinous carcinoma ( $n = 1$ ). The tumor samples were immediately fixed in 4% buffered formalin for maximally 24 hours and then routinely processed for paraffin embedding. These paraffin-embedded tumor tissues were used for the trivariate FCM analysis in parallel with immunohistochemistry.

### Preparation of Cell Suspension

From each of the routinely processed paraffin-embedded tumors, two 50- $\mu$ m-thick sections were cut. These were deparaffinized in xylene and rehydrated in a descending ethanol series. The sections were then immersed in cold citrate solution (2 mg citric acid/mL aqua dest, pH = 6.0) and placed at 80°C in a water bath for 2 hours. After a 15-minute cooling period at room temperature, the sections were rinsed in phosphate-buffered saline (PBS; pH 7.4). The sections were digested for 10 minutes at 37°C in a solution of 1 mg/mL pepsin (Sigma, St Louis, MO) in 0.1 N HCl. The sample was then filtered through a 50- $\mu$ m mesh nylon filter. The cell suspension was centrifugated at 400g, and the pellet was resuspended in PBS, supplemented with 1% bovine serum albumin (BSA; Sigma; BSA/PBS-buffer).

For the study of antibody binding kinetics, 2 different breast tumors were selected and cell suspensions prepared as described. To study the effect of duration of pepsin digestion on immunoreactivity in the single cells, cell suspensions of these 2 tumors were incubated for different periods with the pepsin solution (ie, 5, 10, 20, 30 minutes).

To study the effect of incubation time with the primary antibody on signal intensity, single cells were incubated for different periods with the primary antibodies (ie, 1, 3, 16, and 40 hours) at room temperature.

To evaluate the reproducibility of the FCM assay, that is, the intra-assay and interassay variability, 5 50- $\mu$ m-thick consecutive paraffin sections of 5 different breast tumors were split into 2 parts. One part of each tumor sample was analyzed for cytokeratin-, ER-, and PR-immunoreactivity within 1 assay, whereas the other part was analyzed in 5 separate assays.

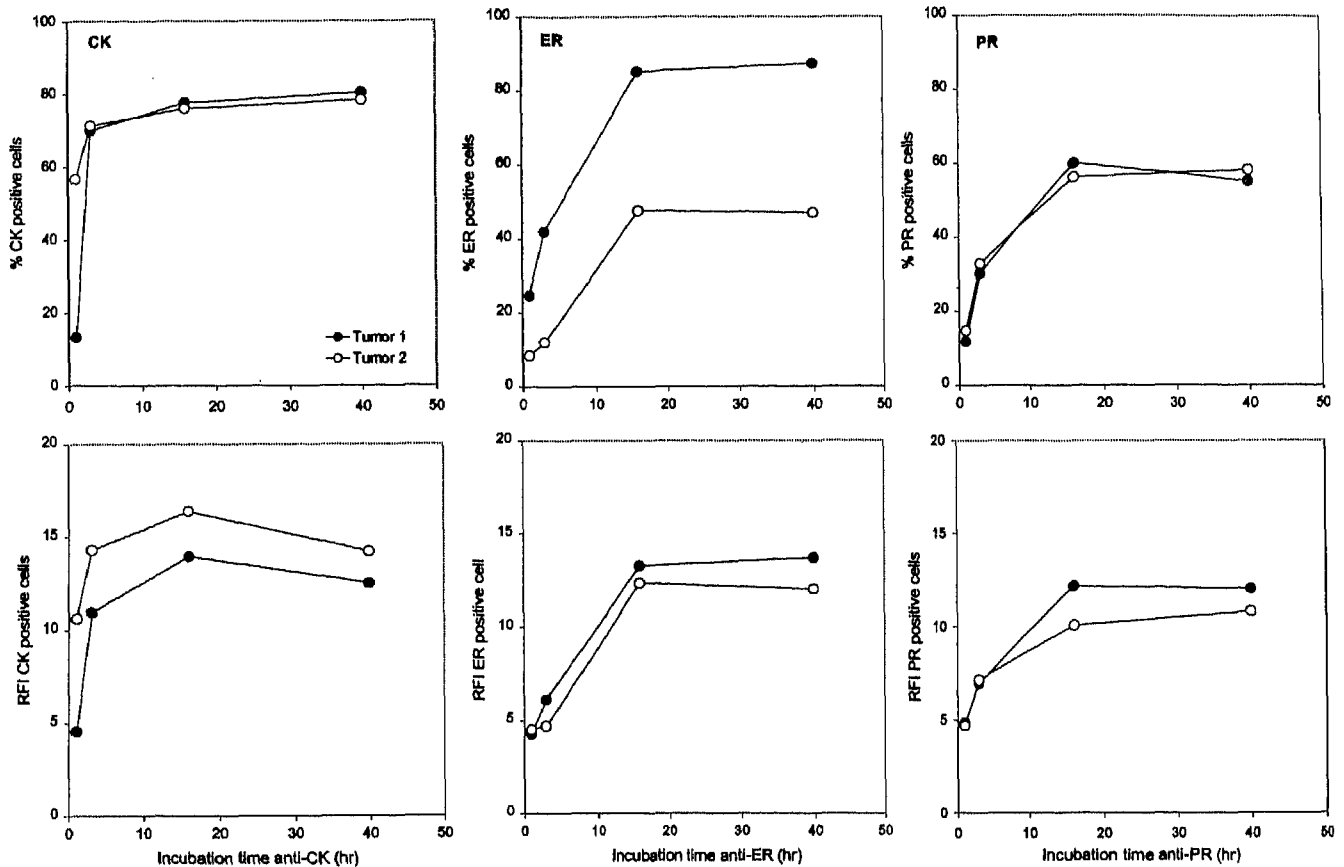
### Multiparameter Flow Cytometry

The double indirect fluorochrome-labeled antibody technique with fluorescein isothiocyanate (FITC)- and r-phycoerythrin (RPE)-conjugated secondary step reagents was applied in the multiparameter FCM analyses. The characteristics and sources of the primary antibodies used are summarized in Table 1. The single cell suspension was aliquoted into 100- $\mu$ L samples. To each sample, 2 primary antibodies appropriately diluted were added simultaneously, that is, the polyclonal cytokeratin antiserum in combination with a monoclonal antibody to one of the steroid hormone receptors (ER or PR). After overnight incubation at room temperature, the samples were rinsed twice in PBS. Binding of primary antibodies was detected by incubating the cell pellet simultaneously with 2 secondary antibodies, that is, goat anti-mouse immunoglobulin (Ig)-FITC (DAKO A/S, Glostrup, Denmark, diluted 1:10) and goat-anti-rabbit-Ig-RPE (Boehringer Mannheim, NL, diluted 1:12). After incubation for 1½ hours at room temperature, the samples were rinsed twice in PBS, and finally the DNA was stained with propidium iodide (PI; 1.0  $\mu$ g PI/mL, Sigma) in PBS containing 0.1 mg/mL RNase (Serva, Heidelberg, Germany). The samples were allowed to stand for 15 minutes on ice in the dark before FCM analysis. As negative controls for background staining, a cell suspension was simultaneously incubated with mouse monoclonal anti-BrdU (clone IIB5, diluted 1:50; as a control for the mouse primary antibodies) and with a polyclonal antiserum directed against bovin mycobacterium (DAKO A/S, diluted 1:800; as a control for the rabbit primary antiserum).

All samples were analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cells were excited with a single 488-nm Argon laser. FITC fluorescence was detected through a 515- to 545-nm BP filter, RPE-fluorescence through a 572- to 588-nm BP filter, and PI fluorescence through a 572- to 588-nm BP filter. Electronic gating was used to exclude doublets and cellular debris. At least 20,000 events were collected for each sample. FITC and RPE signals were recorded as logarithmic amplified data, and the PI-signals were recorded as linear amplified data. The following settings were

**TABLE 1.** Characteristics of Primary Antibodies Used in This Study

Antigen	Antibody	Ig Subtype	Tissue Section	Cell Suspension	Reference	Source
Cytokeratin	Rabbit polyclonal	—	1:500	1:500	32	DAKO A/S, Glostrup, Denmark
Mycobacterium	Rabbit polyclonal	—	1:800	1:800	33	DAKO A/S, Glostrup, Denmark
ER	1D5	IgG <sub>1</sub>	1:100	1:20	34	DAKO A/S, Glostrup, Denmark
PR	1A6	IgG <sub>1</sub>	1:100	1:20	19	DAKO A/S, Glostrup, Denmark
BrdU	IIB5	IgG1	1:100	1:50	35	Eurodiagnostica, Arnhem, NL



**FIGURE 1.** Effect incubation time on primary antibody binding (cytokeratin, ER and PR) as determined by the percentage of positive cells (A) as well as the relative fluorescence intensity (B) of the immunoreactive cells. An optimal staining as determined by saturation is achieved after an incubation time of 16 hours.

used: 407 V, 329 V, and 354 V on photomultiplier tubes for FL1 (FITC), FL2 (PE), and FL3 (PI), respectively. Compensation for (FL1-%FL2), (FL2-%FL1), (FL2-%FL3) and (FL3-%FL2) was 0.9%, 39.8%, 41.8% and 8.3%, respectively. Data analysis was performed using Lysis II software (Becton Dickinson). For cell cycle analysis, Modfit LT2.0 (Verity Software House, Inc. Maine) was used. The following data were collected: 1) the DNA index of the tumor cells and the CV of the first  $G_0/G_1$  peak; 2) the S-phase fraction (SPF); 3) the percentage of ER- or PR-positive cells; and 4) the relative fluorescence intensity (RFI) of the hormone receptor signal in the cytokeratin-positive fraction of the tumor.

The number of hormone receptor-positive cells was determined in the cytokeratin-positive fraction by an arbitrary threshold setting allowing 5% of positive counts in the negative control. Concerning the SPF, tumors were divided into 3 categories: tumors with a low SPF (less than 10%), tumors with an intermediate SPF (10% to 25%), and tumors with a high SPF (more than 25%). The RFI was calculated as the ratio of the mean fluorescence signal of the cytokeratin-positive cell population of the sample as compared with that of the negative control.

#### Steroid Hormone Receptor Immunohistochemistry on Paraffin Sections

In parallel to steroid hormone receptor staining in suspension, consecutive sections 4  $\mu$ m thick were cut from the same paraffin-embedded blocks. They were mounted on 3-aminopropyltriethoxy silane (APES)-coated slides and air-

dried overnight at 37°C. For immunostaining, the sections were deparaffinized in xylene and rehydrated in a descending ethanol series. Endogenous peroxidase activity was blocked by immersion for 10 minutes in 3% hydrogen peroxide in methanol, after which the slides were rinsed in PBS (pH = 7.2-7.4). The slides were placed in a 0.1 mol/L citrate buffer (pH = 6.0) and boiled for 10 minutes in a microwave oven at 750 W. After preincubation with 1% BSA (Sigma)/PBS for 10 minutes, the same monoclonal antibodies directed against the ER and PR were applied at the appropriate dilution (Table 1) overnight at room temperature (RT). As a negative control, sections were incubated with anti-BrdU. Tissue sections of an ER- and PR-positive human mammary carcinoma served as a positive control. Next to this, the intraductal epithelial glands served as an internal positive control. After washing in PBS, the secondary antibody (biotin-labeled goat anti-mouse Ig; 1:400 diluted; DAKO A/S) was applied for 45 minutes at RT. After washing in PBS, these slides were incubated with streptavidin conjugated with horseradish peroxidase (1:600; DAKO A/S). After washing in PBS, peroxidase activity was detected with 3,3'-diaminobenzidine/-0.002%  $H_2O_2$  solution (Sigma). Finally, the sections were counterstained with Harris' hematoxylin, dehydrated, cleared in xylene, and finally mounted in Entellan (Chroma Gesellschaft; Schmid GmbH & Co, Berlin, Germany). To minimize the effect of factors that influence the staining intensity, all sections were stained within 1 run. The intensity and distribution of the specific staining reaction for ER and PR in the breast carcinoma samples were evaluated independently by 2 observers. Immu-

nostaining in less than 10% of the tumor cells was categorized as 0, staining in 10% to 30% of tumor cells as 1, staining in 30% to 60% of the tumor cells as 2, and staining in more than 60% of the tumor cells as 3. The staining intensity was categorized 0, 1, and 2 (negative-weak, moderate, and strong, respectively). The total immunostaining score consisted of the product of the outcome in both categories, resulting in a minimum score of 0 and a maximum of 6.

## RESULTS

### Methodological Improvements

Heating of dewaxed and rehydrated 50- $\mu$ m-thick tissue sections for 2 hours at 80°C in a citrate solution, followed by a mild pepsin digestion of 15 minutes at 37°C, resulted in a high cell yield with little debris and few cell clusters. As observed by immunofluorescence microscopy, the recovered cells showed an intact nuclear morphology, and ER and PR immunoreactivity appeared to be localized in the nuclei. Furthermore, the cells had retained enough cytoskeletal remnants to allow detection of epithelial cells on the basis of anti-cytokeratin immunofluorescence.

In a pilot series, it was noticed that the hormone receptor-positive cells showed a stronger immunofluorescence reaction after overnight incubation with the antibodies directed against ER and PR as compared with a short incubation of 1 hour at room temperature.

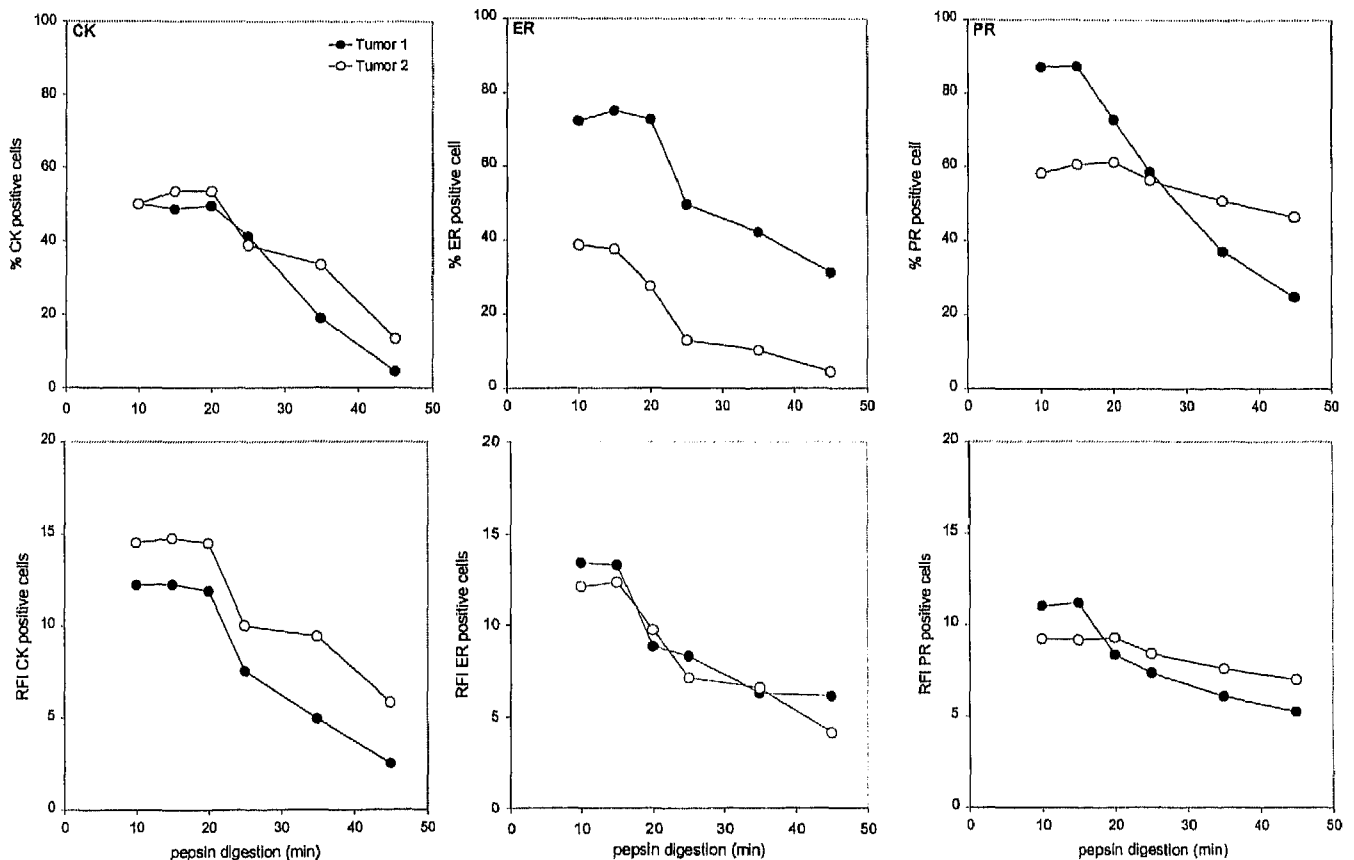
When the kinetics of antibody binding were investigated in more detail, optimal binding for all antibodies used was seen after approximately 16 hours of incubation at RT (Fig 1).

To investigate whether proteolytic digestion interfered with the semiquantitative measurement of hormone receptor content, single-cell suspensions were treated with pepsin for an additional period. As depicted in Figure 2, prolonged proteolytic treatment influenced the levels of hormone receptor content as measured by ER or PR immunoassays, as well as for cytokeratin. A digestion step longer than 15 minutes resulted in a rapid decrease in immunoreactivity.

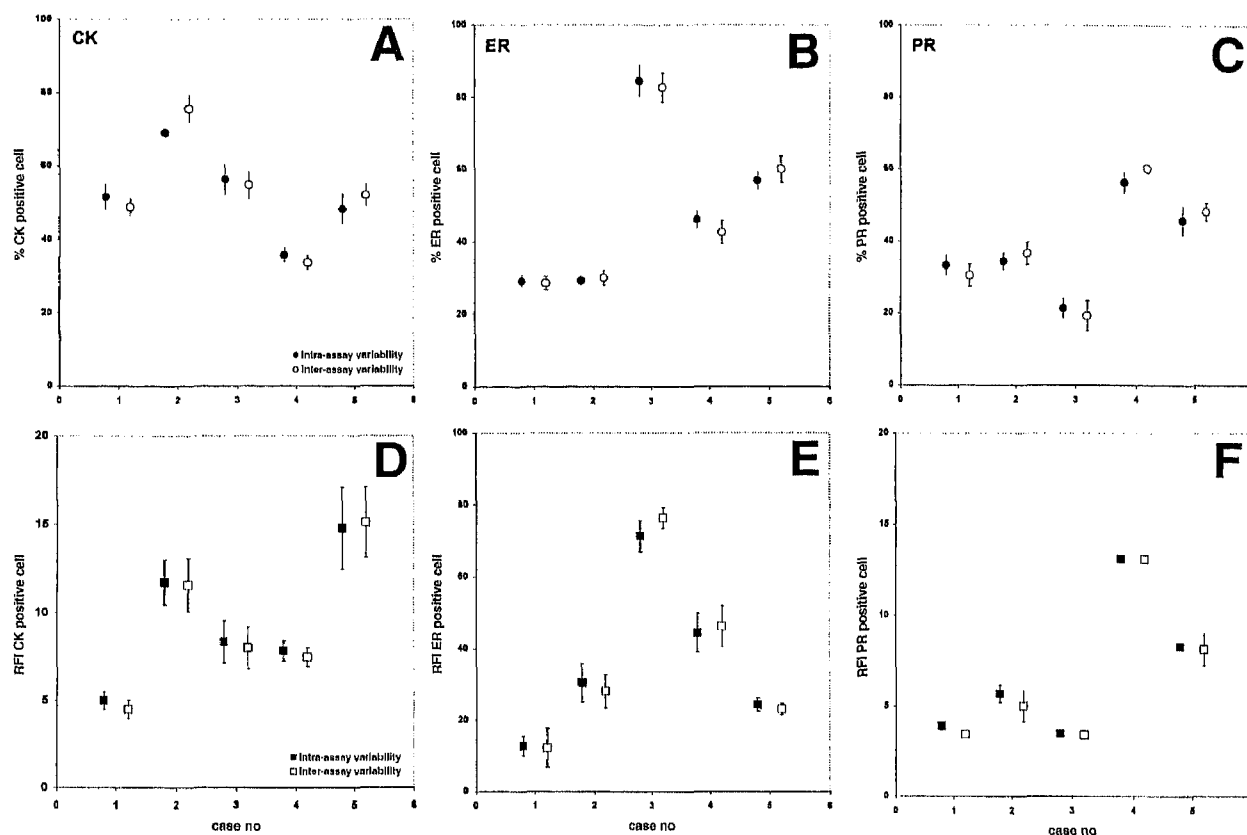
To determine the reproducibility of the FCM assay, that is, the intra-assay and interassay variability, 5 intact consecutive sections of 5 different tumor samples were split into 2 parts. One part was analyzed for CK-, ER-, and PR-immunoreactivity within 1 assay, and the other part was analyzed in 5 separate assays. Figure 3 shows that both the intra-assay and interassay variability is less than 6%.

### Trivariate FCM Analysis of Breast Cancers: Comparison With Immunohistochemistry on Tissue Sections

A total of 42 breast carcinomas were analyzed for the simultaneous expression of cytokeratin and ER or



**FIGURE 2.** Effect of proteolytic digestion on immunoreactivity for cytokeratin, ER and PR. A proteolytic treatment for longer than 15 minutes results in a rapid decline of all antibodies in the number of positive cells as well as the staining intensity as indicated by the RFI.



**FIGURE 3.** Intra-assay and inter-assay variability of trivariate cytokeratin/hormone receptor/DNA FCM analysis. Intra-assay variability was determined by duplicate analysis of 5 consecutive tissue sections of the same tumor sample within 1 assay. Interassay variability was determined by analysis of 5 consecutive tissue sections of the same tumor sample in 5 separate assays. The mean number and standard deviation (SD) of positive cells for cytokeratin, ER, and PR are shown in a, b, and c, respectively, whereas the relative fluorescence intensity (RFI) and SD is shown in d, e, and f.

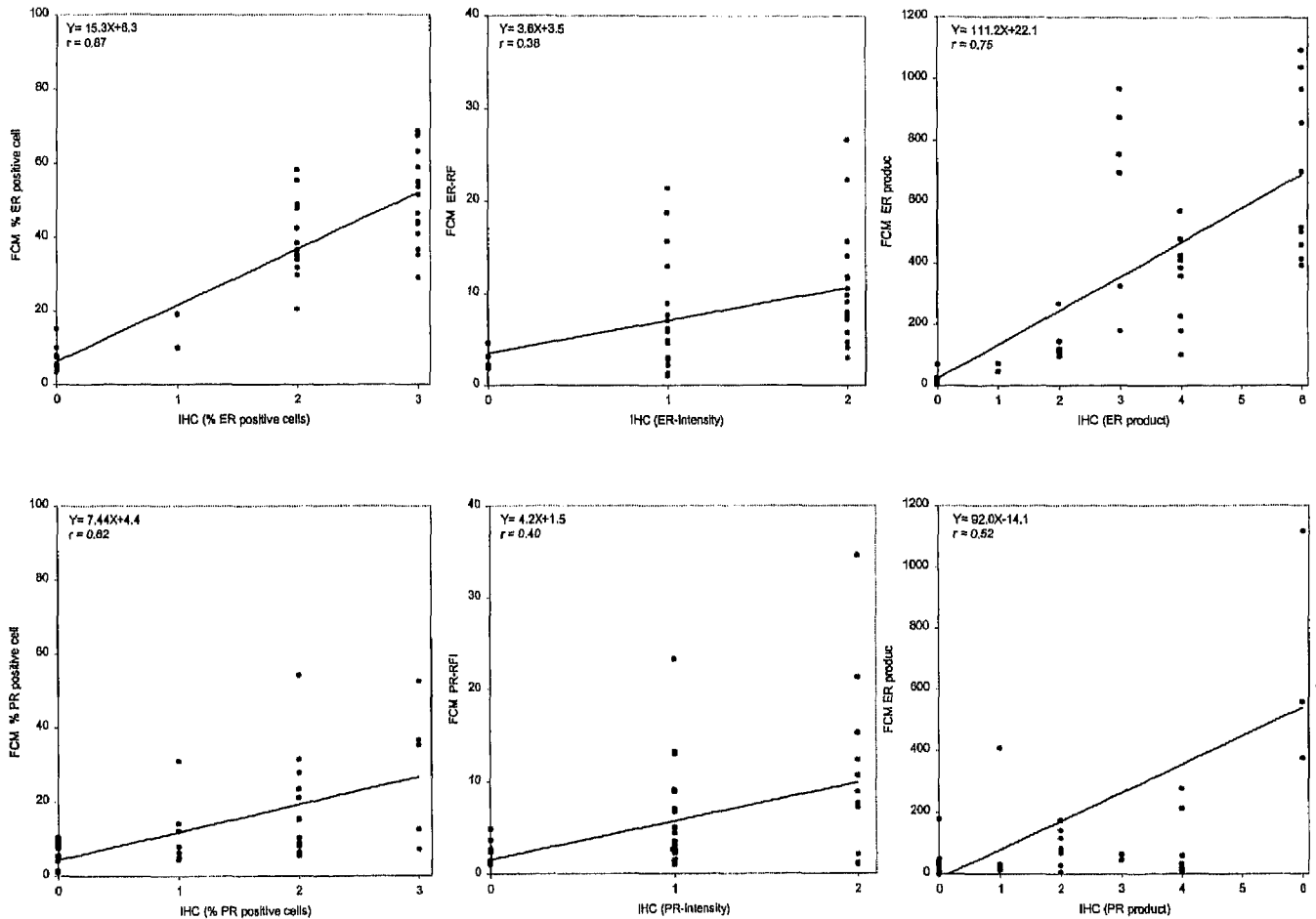
cytokeratin and PR, next to DNA content. When the steroid hormone receptor-positive and steroid hormone receptor-negative cases were grouped separately, a good correlation between immunohistochemistry and FCM was found for ER receptor content (Table 2). Thirty-two cases were positive for both assays. Eight of 42 breast carcinoma samples were found negative with both assays. Two cases that were negative for ER, as determined by immunohistochemistry, contained a small percentage of strongly ER-positive normal duct epithelial cells. When analyzed by FCM, these ER-positive cells were DNA-diploid cells, whereas the DNA-aneuploid tumor cells were devoid of ER staining. These 2 samples were therefore classified negative in both assays. For PR receptor content analysis, a discrepancy was found in 2 of 42 cases. Both cases were scored positive with immu-

nohistochemistry, whereas the FCM analysis showed less than 5% positive cells. In both cases, immunohistochemistry showed low numbers of positive cells with very low levels of immunoreactivity. In all other cases a good correlation was found.

To investigate the correlation between the 2 methods, that is, immunohistochemistry and FCM, in more detail, the percentage of steroid hormone receptor-positive epithelial cells, as determined by FCM, was compared with the semiquantitative immunohistochemical score of ER- or PR-stained tissue sections (Fig 4). For ER as well as PR, a statistically significant correlation ( $r = .87$  and  $r = .62$ , respectively,  $P < .0001$ ) was found between the fraction of hormone receptor-positive cells as determined by FCM and immunohistochemistry. Next to the percentage of hormone receptor-positive epithelial cells, the RFI of the steroid hormone receptor immunostaining as determined by FCM after selection of the cytokeratin-positive cells was plotted against the staining intensity of the hormone receptors as determined by the immunohistochemical score (Fig 4). No correlation between RFI and the immunohistochemistry was found. In addition to the quantification of hormone receptor-positive epithelial cells (for an example, see Fig 5), the DNA histograms (mean CVs = 3.02%) also allowed accurate DNA-ploidy deter-

**TABLE 2.** Comparison of the Results of Immunohistochemistry and Flow Cytometry for ER and PR in Paraffin-Embedded Breast Carcinomas

FCM	ER-IH			PR-IH		
	Negative	Positive	Total	Negative	Positive	Total
Negative	10	0	10	15	2	17
Positive	0	32	32	0	25	25
Total	10	32	42	15	27	42



**FIGURE 4.** Comparison between Immunohistochemistry and FCM analysis for presence of ER (A) and PR (B). The left graph on each row shows the correlation between the fraction of steroid hormone receptor-positive cells as determined by Immunohistochemistry and FCM (in the cytokeratin-positive compartment). Although somewhat weaker for PR, a significant correlation ( $P < .0001$ ) was found for both steroid hormone receptors. The middle graph shows the correlation between the intensity of the steroid hormone receptor staining as determined by the 2 methods. No correlation was found here. The right graph finally shows the product of the fraction of steroid hormone receptor-positive cells and the intensity of the staining of the Immunohistochemistry and the FCM analysis.

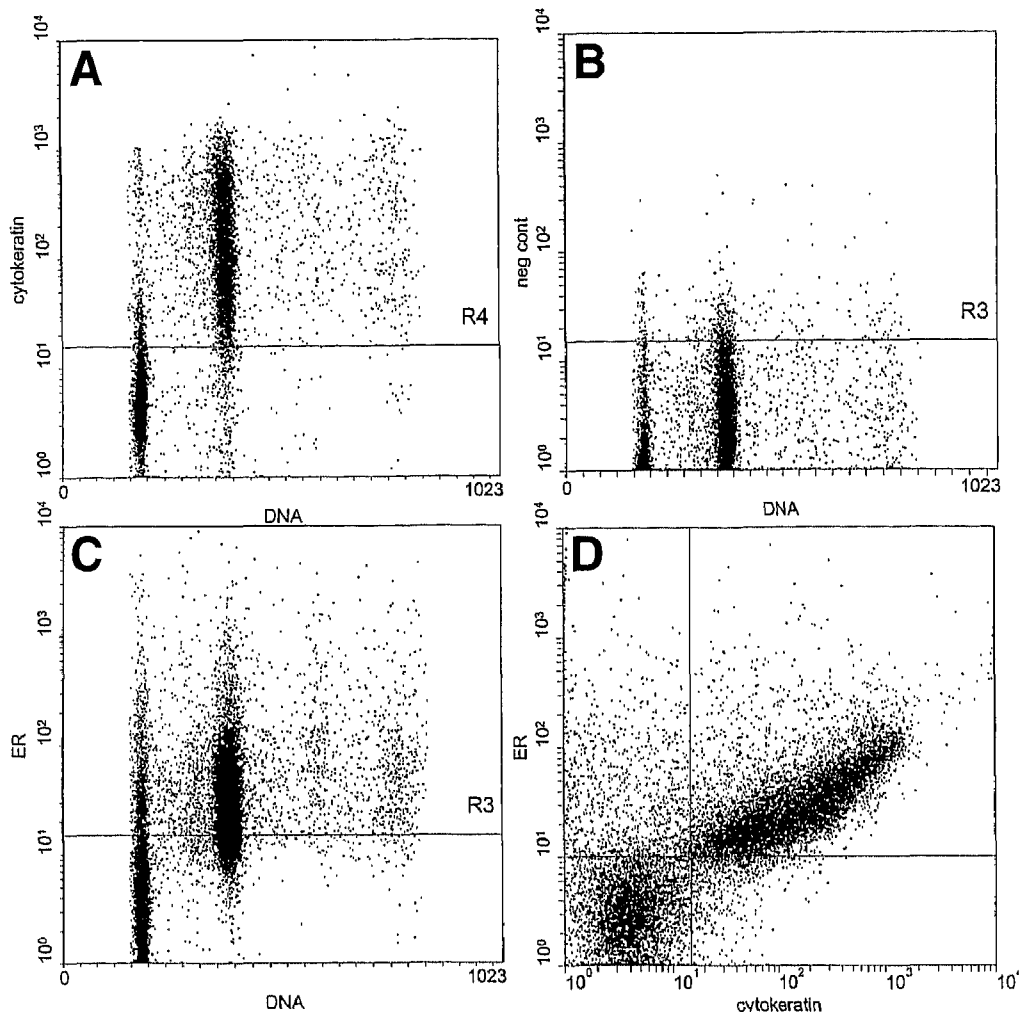
minations and cell cycle analyses. In this way, the steroid hormone receptor reactivity of the relevant epithelial (tumor) cells could be correlated directly to the ploidy status and the growth potential of these cells (Fig 6). It became clear from Figure 6A that 3 subpopulations of tumors could be recognized:

- Group 1: Tumors with a small fraction of ER-positive cells (less than 20%) and an intermediate and high SPF
- Group 2: An intermediate group of tumors with a relatively high percentage of ER-positive cells and an intermediate SPF
- Group 3: Tumors with a great fraction of ER-positive cells (more than 30%) and simultaneously a low SPF (less than 10%).

When comparing these results with the expression of PR, it appears that almost half of the tumors of group 3 had also a large fraction of PR-positive cells, whereas all tumors of group 1 lacked expression of PR. All but 2 tumors from group 2 had also a small fraction of PR-positive cells.

## DISCUSSION

From previous studies, it is clear that parameters such as DNA ploidy, steroid receptor content, and cell cycle characteristics each bear prognostic information in breast carcinoma,<sup>2,4-11,13,20</sup> but this information has not been used to its full extent because of technical limitations or the composition of the material. For instance, single-parameter DNA flow cytometry has frequently been used to determine ploidy levels and cell cycle characteristics, but this approach is hampered by the fact that tumor biopsy specimens not only consist of tumor cell but actually contain a heterogeneous mixture of epithelial cells (normal and tumor), fibroblasts, and vascular stromal components, as well as inflammatory cells. In this way, small subsets of DNA-aneuploid cells escape detection, and more importantly, cell cycle characteristics are often not informative, for instance, in cases of DNA diploidy or in case of insufficient peak separation in DNA-peridiploid tumors. This drawback applies to cell suspensions derived from both fresh and



**FIGURE 5.** An example of a FCM analysis of a tetraploid breast tumor with a high expression of estrogen receptors (ER). Cells were stained in suspension for cytokeratin, ER, and DNA. A shows the dotplot of DNA (x-axis) versus cytokeratin. B and C show a dotplot of DNA content (x-axis) versus negative control and ER, respectively. The solid lines denote the threshold for positivity based on the negative control (B). D finally shows the dotplot of cytokeratin (x-axis) versus ER. From A it became clear that most cytokeratin-positive cells are tetraploid. Note that these cytokeratin-positive cells are also positive for ER, whereas cytokeratin-negative cells are negative for ER (D).

paraffin-embedded tissues, but now can be overcome by selection of epithelial cells for ploidy measurements by means of bivariate cytokeratin/DNA analysis.<sup>14-16</sup> Determination of steroid hormone receptor content is hampered by similar drawbacks.<sup>12</sup> Quantitative data on steroid hormone receptor content can be obtained with the cytosol-based ligand-binding assays.<sup>10</sup> However, these techniques do not compensate for the presence of nonrelevant cells, such as stromal and inflammatory cells, in the sample. This inherent drawback has been circumvented by the immunocytochemical detection of steroid receptors in tissue sections or cytological preparations.<sup>2,21,22</sup> This approach, however, is sensitive to many external factors interfering with accurate quantification of receptor content. For instance, staining intensity is influenced by the duration of fixation, thickness of the tissue section, incubation conditions of the primary antibodies, concentration of the chromogens, and often subjective scoring of the investigators.

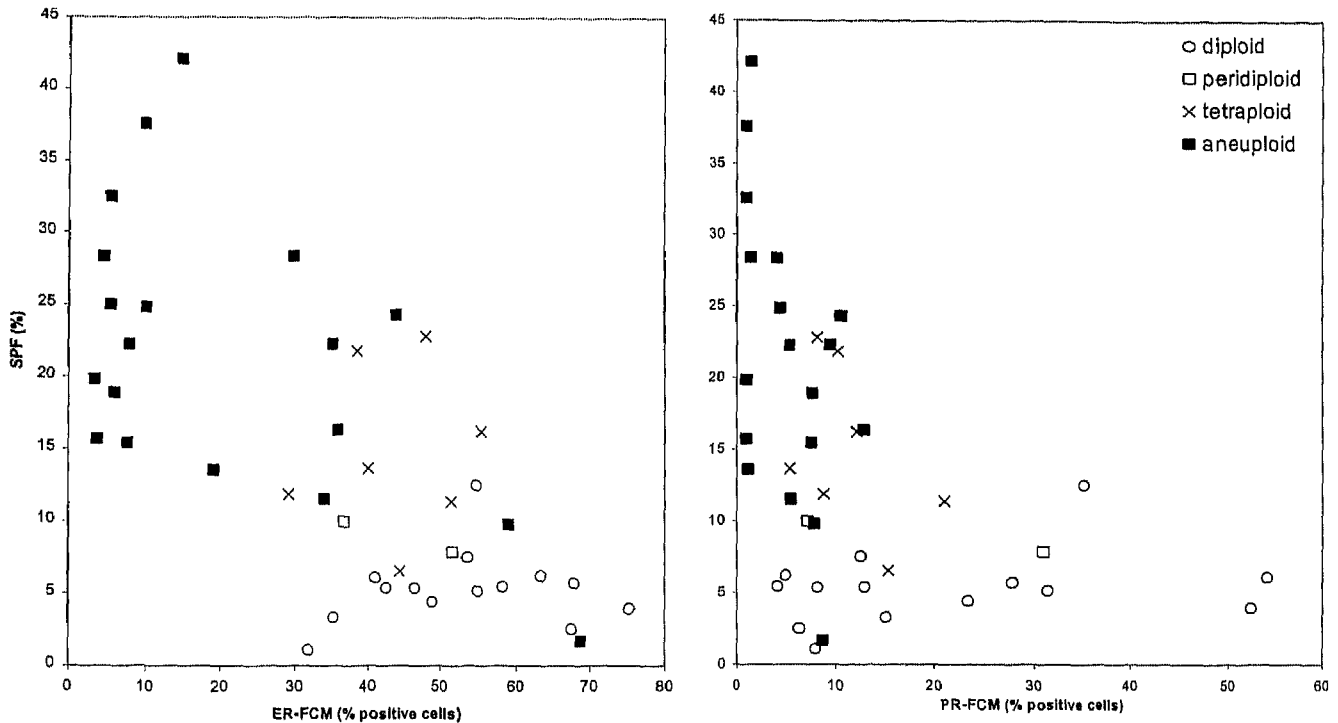
Despite these limitations all individually determined parameters apparently bear prognostic relevant information. However, it is reasonable to assume that accurate and combined analysis of ploidy level, steroid receptor content, and cell cycle characteristics of the relevant epithelial cells will add to this information and might allow the detection of specific subsets of tumors

and tumor cell populations and help to predict the individuals response to therapy.

In the underlying study, we applied our recently developed protocol for the multiparameter FCM analysis of formalin-fixed, paraffin-embedded material<sup>17</sup> to a series of routinely processed breast carcinoma specimens. The aim of the study was to investigate the applicability of this protocol for the accurate enumeration of steroid receptor-positive, epithelial (tumor) cells, and to explore whether this multiparameter approach allows the detection of specific, clinically relevant subsets of tumors, based on ploidy level, steroid receptor expression, and cell cycle characteristics.

We previously showed that heat pretreatment of dewaxed and rehydrated tissue sections resulted in increased cell recovery, improved quality of the obtained DNA histograms, and restoration of epitopes that were masked during formalin fixation.<sup>17</sup> Here we show that this improved protocol also allows reliable detection of steroid receptor content. First, proteolytic digestion up to 15 minutes results in (1) sufficient recovery of cells, (2) steady levels of cytokeratin, estrogen and progesterone hormone receptor immunofluorescence, and (3) high-quality DNA histograms. Second, antibody-binding kinetics indicate that overnight incubations at room temperature result in saturated





**FIGURE 6.** The relationship between ploidy, SPF, and estrogen receptor or progesterone receptor status as determined by FCM. The closed squares represent the aneuploid, the open squares the peridiploid, the asterisks the tetraploid, and the open circles the diploid tumors. From A it became clear that 3 subsets of patient populations could be recognized. First, tumors with intermediate and high SPF, almost complete absence of ER, and all aneuploid. Second, tumors with intermediate SPF and a relatively high percentage of ER-positive cells. This group includes almost all tetraploid and peridiploid tumors. The third group is characterized by tumors with a low SPF, high percentage of ER-positive cells, and included all diploid tumors.

antibody binding. Third, intra-assay and interassay variability is low, that is, less than 6%. Finally, a significant correlation was found between the immunohistochemically determined fraction and the FCM-derived percentage of steroid hormone receptor-positive cells. No correlation was found between the immunohistochemical and flow cytometric determined staining intensity of steroid hormone receptors. This finding is somewhat surprising, because we previously showed for cell lines that fluorescence intensity correlated well with the number of hormone-binding sites.<sup>12</sup> Therefore, we conclude that routinely determined immunohistochemical scores are based more on numbers of receptor-positive cells than on the staining intensity, which is difficult to interpret by the (subjective) human eye.

Because FCM analysis is performed on a per-cell basis, admixture of receptor-negative and receptor-positive cells has no influence on the fluorescence level of individual cells. Where cytosolic-based assays lack morphological information, our approach always has a sequential hematoxylin and eosin (H&E)-stained section available to support the correct interpretation of cell composition and to trim the 50- $\mu$ m section with a scalpel blade for the most relevant parts. Using the cytokeratin antibodies to specifically identify the epithelial cells in this selected area enables additional specification of the cells that are submitted for receptor assay. The FCM equipment guarantees the objective single-cell quantification as receptor positive or negative. In the case of a diploid tumor, it is not possible to

distinguish the tumor cells by their DNA profile from the normal duct epithelial cells. Fortunately, immunocytochemical studies have shown that immunostaining for hormone receptors is sparse (on average, 7% of cells) in the normal breast epithelium, and is largely confined to the lobular elements in the premenopausal breast.<sup>9,23</sup> As mentioned previously, the interpretation of these flow cytometric-generated data can be supported by the information from the already available H&E-stained section.

Although applied to this limited series of routinely processed breast tumors, multiparameter FCM analysis showed 3 discrete subsets of patients based on the fraction of ER-positive cells, SPF, and ploidy. The first subgroup consists of patients with DNA-aneuploid tumors with intermediate and high SPF and almost complete absence of ER-positive cells. A second subgroup showed DNA-aneuploid tumors, including all tetraploid and peridiploid ones, with intermediate SPF and a relatively high percentage of ER-positive cells. The third subgroup consists of tumors with low SPF and high percentage of ER-positive cells. Most (13 of 16 cases) of the tumors in the latter subgroup were DNA diploid. With the exception of 2 cases, all tumors showing more than 20% PR-positive cells belonged to group 3. From a cell biological point of view and based on reports from the literature, these subsets of patients are likely to respond better to therapy and therefore represent a prognostically separate entity.<sup>24-29</sup> The 2 exceptions mentioned both belong to group 2 based on

their SPF, which was slightly more than the arbitrary 10% threshold. One of them was diploid and the other tetraploid. They may have to be considered as transitional cases between group 2 and 3. Long-term follow-up of a well-documented cohort of patients is needed for a definitive answer as to the prognostic significance of multiparameter FCM. The method presented in this article makes such an approach feasible because it is applicable to routinely fixed and formalin-embedded material. Furthermore, preliminary experiments suggest that, except for steroid hormone receptors, other cell constituents such as membrane antigens (eg, E-cadherin)<sup>30</sup> and apoptosis-specific markers such as C-terminally cleaved cytokeratin 18 fragments,<sup>31</sup> can be detected in these cell suspensions. This allows rapid screening of archival material for the prognostic significance of biological determinants such as invasiveness and apoptosis, in relation to cell cycle characteristics and ploidy.

In conclusion, multiparameter FCM of formalin-fixed, paraffin-embedded tissues allows accurate and combined analysis of ploidy level, percentage of steroid receptor-positive cells, and cell cycle characteristics in the relevant epithelial tumor compartment. By the combined assessment of these biological determinants, specific subsets of patients can be identified that would otherwise escape detection. This approach might be of help in the fine tuning of specific therapy for individual patients.

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